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Determination of Assay and Peak Purity Evaluation of Acyclovir and Valacyclovir by RP-HPLC Method

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Abstract: An isocratic reversed-phase HPLC method with PDA detector has been developed for the assay and purity evaluation of Acyclovir and Valacyclovir in bulk drug. The assay and purity of Acyclovir and Valacyclovir was analysed. The analysis was performed using inertial cyno column (250 x 4.6mm, 5µ) as a stationary phase with column oven temperature 45°c and UV detection at 254nm. The separation was achieved using isocratic program of buffer (Buffer used was of 0.1% Ammonium acetate in water) and Acetonitrile in the ration 95:5. The method was optimized based on the peak shapes and resolution of Acyclovir, Acyclovir Imp A, Valacyclovir and Valacyclovir related imp c. The method was validated as per International Conference of Harmonization (ICH) guidelines in terms of linearity, precision, accuracy, specificity, robustness and solution stability. The sample concentration were injected was 2 mg/ml and 5 mg/ml for Acyclovir and Valacyclovir respectively. The method is linear within the range of 100 to 300 µg/ml for acyclovir and 250 to750 µg/ml for Valacyclovir.

Keywords: Acyclovir; Valacyclovir, RP-HPLC.

I. INTRODUCTION

Acyclovir {9-[(2-hydroxyethoxy) methyl] guanine, zovirax} is a guanosine analogue with an acyclic side chain at the 9 position. It is a prototype of the group of viral agents that are activated by viral thymidine kinases (Tk) to become inhibitors of viral DNA polymerases and block viral DNA synthesis Acyclovir uptake and intracellular phosphorylation to monophosphate is mediated by viral thymidine [1]. Acyclovir is an acyclic guanosine derivative which exhibits a selective inhibition of herpes viruses replication with potent clinical antiviral activity against the herpes simplex and varicella zoster viruses. As acyclovir is structurally similar to endogenous substances, its analysis in human serum is complicated and requires high selective analytical methods. Immunological techniques and HPLC are the most common used methods for determination of acyclovir in biological samples. Acyclovir is a nucleoside analog with antiviral activity against herpes viruses. This drug is an effective agent in the treatment of herpes virus infections and may also used in the prophylaxis of cytomegalovirus infections in immune compromised patients. Some HPLC methods for the analysis of acyclovir in plasma, serum or urine has been published [2, 5]. However, these analytical methods require the use of an ion-pairing agent [6] column thermostating and fluorimetric detection [7] or are limited by the lack of sensitivity . Several HPLC methods [8, 9] have been published for determination of acyclovir in human serum using UV or fluorescence detection. Since acyclovir is a polar compound and soluble in aqueous medium and practically insoluble in most of organic solvents, protein precipitation with perchloric acid [10,11] or solid phase extraction [12] are applied for pre-treatment of the drug in serum samples. While the sensitivity of analysis is significantly reduced due to dilution of the samples after deproteinization, injection of the acid supernatant after precipitation of proteins by perchloric acid leads to numerous late eluting peaks and significant reduction of the lifetime of analytical column. Time consuming gradient elution is needed for removing of the late-eluting peaks, and deterioration of column performance significantly reduces the number of samples which can be analyzed.

II. MATERIAL AND METHODS

A. Drug and reagents

Pure Acyclovir and Valacyclovir was obtained as gratis sample from Cipla ltd Research Laboratories (Mumbai, India). Analytical reagent (AR) grade ammonium Acetate was purchased from Fluka (Banglore, India) and Acetonitrile from sigma Aldrich (Mumbai, India). Water for HPLC studies was obtained from milipore water purifying system.

B. Apparatus and equipment

LC was carried out on Waters HPLC system (Model no. 2690) with photodiode array detector (Make-996). The output signal was monitored and processed using Empower software . In all the studies, separations were achieved on a Inertsil Cyno (250 mm x 4.6 mm i.d., particle size 5 µm) procured from LCGC (Banglore, INDIA). Other small equipment were PCI sonicator (22L500/CC/DTC made in), precision analytical balance (Mettler Toledo, Schwerzenbach, Switzerland).

C. Chromatographic conditions

The numbers of column such as waters symmetry C18 (250 x 4.6mm, 5.0µm), YMC packpro C18 (250 x 4.6mm, 5.0µm) Inertsil ODS 3V (250 x 4.6mm, 5.0µm) were used during method development. The separation was achieved using Isocratic program of solution A (i.e Solution A used Contains 0.1% Ammonium Acetate in water): and Solution B is Acetonitrile in the ratio of 95:5 v/v. the flow rate was set at 0.8 ml/min and column was maintained at 45^{o}C. The injection volume was set 3µl and detector was set at a wavelength of 254 nm.

D. Preparation of sample during method development and Validation

The diluent was selected for dissolving Acyclovir and Valacyclovir was mixture of water and Acyclovir (in ration of 50:50 v/v). Standard solution of Acyclovir and Valacyclovir were prepared in diluent having concentration of 0.2 mg/ml and 0.5 mg/ml respectively. Acyclovir and Valacyclovir sample solution were prepared in the concentration of 0.2 mg/ml and 0.5 mg/ml respectively and injected.

E. Preparation of sample for Force degradation studies (Stress testing)

Preparation of sample for Solid state and Liquid state degradation studies: Sample powder treated as given below table and further Acyclovir and Valacyclovir sample solution was prepared in the concentration of 0.2 mg/ml and 0.5 mg/ml respectively and injected.

Sr.No.	Sample condition	FD Condition
1	Elevated	1) 80°C for 48 Hr
2	Photolysis	1) 1.2million Lux Hrs
		2) 200 Watts/hr
3	Acidic	1) 1M HCl for 2Hr
		2) 1M HCl for 1Hr
4	Basic	1) 1M NaOH for 2Hr
		2) 1M NaOH for 1Hr
		3) 0.1M NaOH for 2Hr
5	Oxidation	1) 10% H2O2 for 2Hr
6	Reduction	1) 10% aq. Sodium Bisulphite for 2Hr
		2) 10% aq. Sodium Bisulphite for 1Hr
7	Hydrolysis	1) Purified water

III. Method development and column selection

Chemical structure of Acyclovir, Acyclovir related impurity A, Valacyclovir and Valacyclovir Related comp. c are shown in (Fig.1 to 4). The sample of Acyclovir and Valacyclovir procured from market which was selected for validation studies. Different mobile phase and stationary phases were employed to developed a suitable LC method for the quantitative determination of Acyclovir and Valacyclovir in their respective formulations. A number of column containing various packing materials of ODS supplied by different manufacturers and different mobile phase composition were tried to get good peak shapes and selectivity for the impurities present in Acyclovir and Valacyclovir.

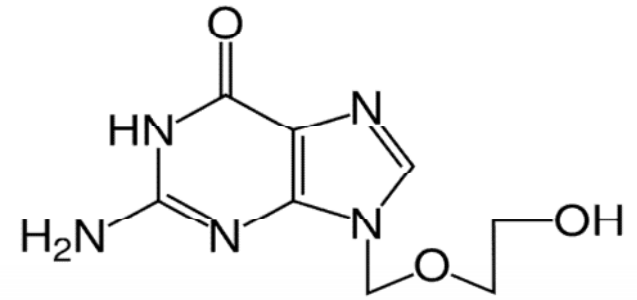


Fig.1. Chemical structure of Acyclovir.

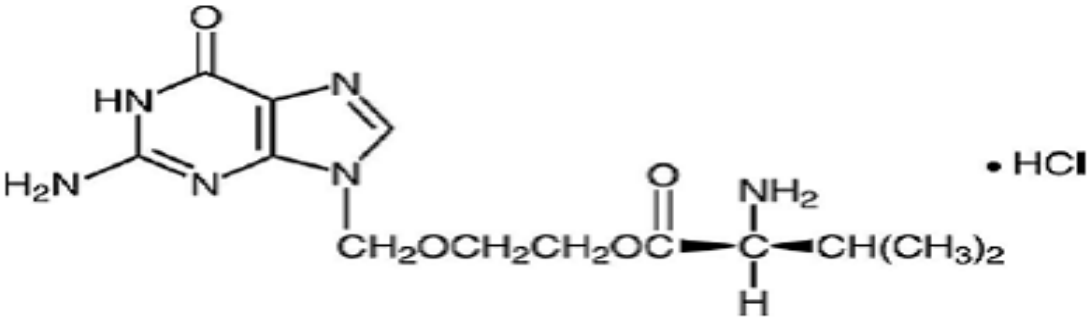


Fig.2. Chemical structure of Valacyclovir.

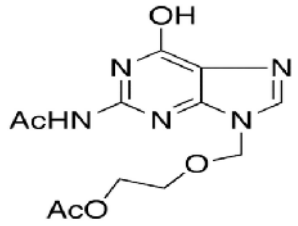


Fig.3. Chemical structure of Acyclovir related Impurity A.

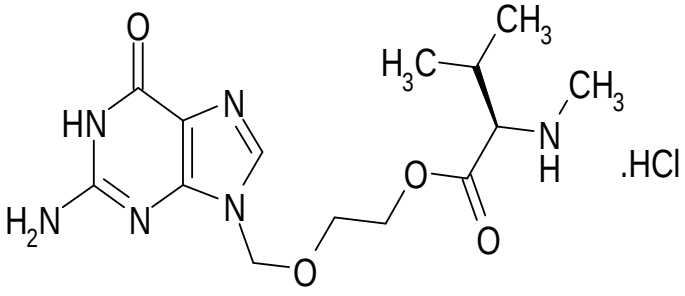
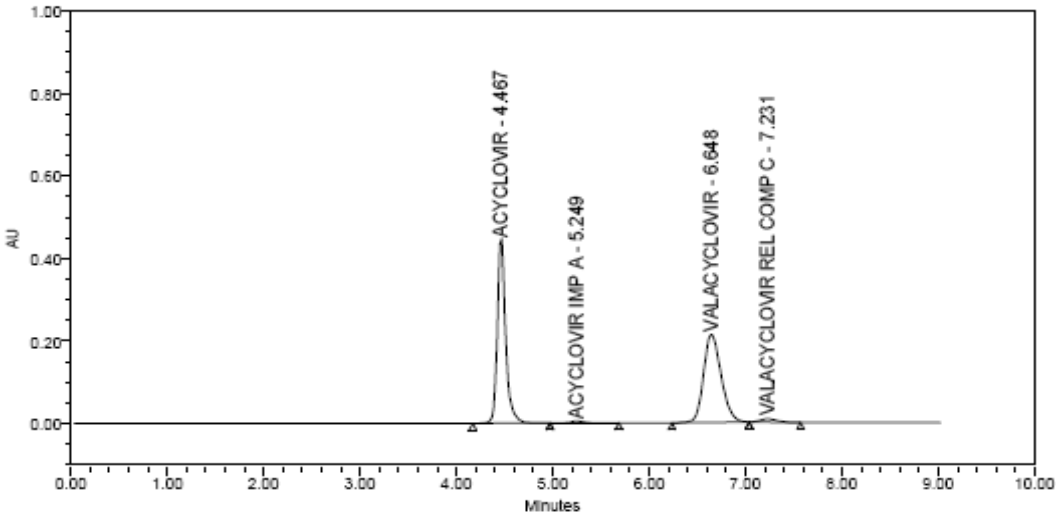


Fig.4. Chemical structure of Valacyclovir related compound C.



	Peak Name	RT	Area	% Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	ACYCLOVIR	4.467	2683907	48.55	446011	14795	1.27	
2	ACYCLOVIR IMP A	5.249	42977	0.78	4395	6795	1.15	3.79
3	VALACYCLOVIR	6.648	2704410	48.92	213390	6308	1.18	4.67
4	VALACYCLOVIR REL COMP C	7.231	97258	1.76	7829	7368	1.34	1.71

Fig.5. Resolution chromatogram of Acyclovir, Valacyclovir and its related Impurities.

Sr. No.	**Mobile phase**	**Stationary Phase**	**Result**
1)	Phosphate buffer, acetonitrile and methanol (50:25:25 v/v/v)	waters symmetry C18 (150 x 4.6mm, 5.0µm) .	Acyclovir Impurity-A and Acyclovir co-eluted
2)	Phosphate buffer, acetonitrile and methanol (50:30:20 v/v/v)	waters symmetry C18 (150 x 4.6mm, 5.0µm) .	Acyclovir Impurity-A and Acyclovir co-eluted
3)	0.1% Phosphate buffer in water: Acetonitrile and Methanol(80:10:10 v/v/v)	phenomenex luna C18 (250 x 4.6mm, 5.0µm)	Peak shape of analytes and impurities was not symmetrical
4)	0.1% Phosphate buffer in water: Acetonitrile and Methanol(80:10:10 v/v/v)	Inertsil ODS (250 x 4.6mm, 5.0µm)	Analyte and impurities co-eluted
5)	0.1% Phosphate buffer in water: Acetonitrile and Methanol(70:15:15 v/v/v)	Inertsil ODS (250 x 4.6mm, 5.0µm)	Peak shape not symmetrical and Analyte and impurities co-eluted
6)	0.1% Ammonium Acetate, Acetonitrile and methanol (75:15:10 v/v/v/)	Inertsil ODS (250 x 4.6mm, 5.0µm)	Analyte and impurities co-eluted
7)	0.1% Ammonium Acetate, Acetonitrile and methanol (75:15:10 v/v/v/)	Inertsil Cyno (250 x 4.6mm, 5.0µm)	Analyte and impurities co-eluted
8)	0.1% Ammonium Acetate and Acetonitrile (75:25v/v/)	Inertsil Cyno (250 x 4.6mm, 5.0µm)	Analyte and impurities co-eluted
9)	0.1% Ammonium Acetate and Acetonitrile (85:15v/v/)	Inertsil Cyno (250 x 4.6mm, 5.0µm)	Analyte and impurities co-eluted
10)	0.1% Ammonium Acetate and Acetonitrile (95:5v/v/)	Inertsil Cyno (250 x 4.6mm, 5.0µm)	Analyte and impurities are well separated and peak shape are symmetrical

The separation was achieved using isocratic program of Buffer (A Buffer used was of 0.1% Ammonium acetate in water): Acetonitrile. The method was optimized based on the peak shapes and resolution of Acyclovir (Fig.1), Acyclovir Imp-A (Fig.3), Valacyclovir (Fig. 2) and Valacyclovir related comp. c (Fig. 4) and for resolution chromatogram refer Fig. 5.

IV. RESULTS AND DISCUSSION

A. Method validation

Specificity: Specificity of the method is its ability to detect and separate all the impurities present in the drug. Specificity of the method is demonstrated in terms of spectral as well as peak purity data of the drug. Peak passed the peak purity test.

Linearity: Linearity of the method was checked by preparing solutions at seven concentration levels of 100 ppm (Level 1) to 300 ppm (Level 7) for Acyclovir and for Valacyclovir the linearity levels are 250 ppm (Level 1) to 750 ppm (Level 7). Level 1 and level 7 was injected six times were as level 2, level 3, level 4, level 5 and level 6 was injected two times. The mean responses recorded for each analyte were plotted against concentration. The correlation coefficient for acyclovir and valacyclovir was found to be 1.00 and 1.00 respectively, which indicates good linearity. (fig. 8 for Acyclovir and fig. 9 for Valacyclovir).

Accuracy: Acyclovir and valacyclovir analytes were spiked in placebo solution at 50%, 100% and 150%. Each spiked solution was prepared in triplicate and injected. The recovery percentage and %RSD were calculated for each analyte. Recovery of acyclovir and valacyclovir ranged from 98.65-100.77% and 99.09-101.47% respectively. The results are shown in Table 1 and 2 respectively.

Table 1. Accuracy Results of Acyclovir.

Added (µg)	Recovered (µg)	%Recovery (Limit NLT 98.0% and NMT 102.0%)	%RSD
100.5	99.5	99.00	98.65
	98.94	98.44	
	98.99	98.50	
208.0	208.19	100.09	100.77
	210.39	101.15	
	210.24	101.08	
305.0	302.65	99.23	99.48
	301.62	98.89	
	305.95	100.31	

Table 2. Accuracy Results of Valacyclovir.

Added (µg)	Recovered (µg)	%Recovery (Limit NLT 98.0% and NMT 102.0%)	%RSD
242.5	241.17	99.45	99.22
	240.78	99.29	
	239.87	98.92	
501.0	506.46	100.69	101.47
	512.53	101.90	
	512.19	101.83	
752.0	744.64	99.02	99.09
	740.28	98.44	
	750.65	99.82	

System and method precision: The system for two impurities in acyclovir and valacyclovir was checked. The sample was prepared by dissolving tablets in diluent of target analyte concentration and injected six times. The %RSD was found to be less than 2.0% for system precision.

To determine the method precision six independent solutions were prepared with respect to target analyte concentration. Each solution was injected once. The variation in the results for the two analytes were expressed in terms of % RSD. The values calculated were found to be below 2.0% RSD for analytes, indicating satisfactory method precision. The results are shown in Table 3.

Table 3. Method Precision Results of Acyclovir and Valacyclovir.

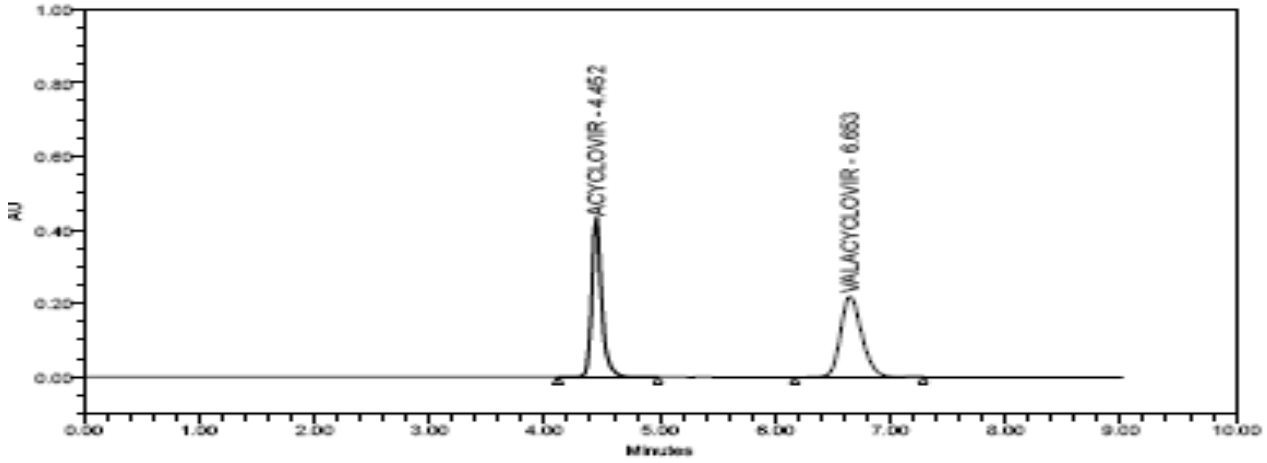
Sr.No.;	Sample Area of Acyclovir	% Recovery of Acyclovir	Sample Area of Valacyclovir	% Recovery of Valacyclovir
Sample-1	**2626688**	98.62	**2884850**	100.06
Sample-2	**2613077**	98.11	**2827733**	98.08
Sample-3	**2647746**	99.41	**2864699**	99.36
Sample-4	**2644364**	99.28	**2863240**	99.31
Sample-5	**2617907**	98.29	**2824606**	97.97
Sample-6	**2608865**	97.95	**2822439**	97.89
Mean		98.61		98.78
SD		0.61		0.92
% RSD		0.62		0.93

Table 4. Solution Stability Results of Acyclovir and Valacyclovir.

Condition	Sample Area of Acyclovir	% Assay of Acyclovir	Sample Area of Valacyclovir	% Assay of Valacyclovir
Sample-0th HR	**2653684**	101.33	**2851206**	99.64
Sample-2nd HR	**2672648**	102.05	**2791826**	97.57
Sample-4th HR	**2694385**	102.88	**2810834**	98.23
Sample-8th HR	**2608865**	99.62	**2822439**	98.64
Sample-12th HR	**2626688**	100.30	**2884850**	100.82
Sample-16th HR	**2662343**	101.66	**2860017**	99.95
Sample-20th HR	**2608865**	99.62	**2822439**	98.64
Sample-24th HR	**2630035**	100.42	**2826126**	98.76
Mean		100.98		99.03
SD		1.19		1.04
%RSD		1.17		1.05

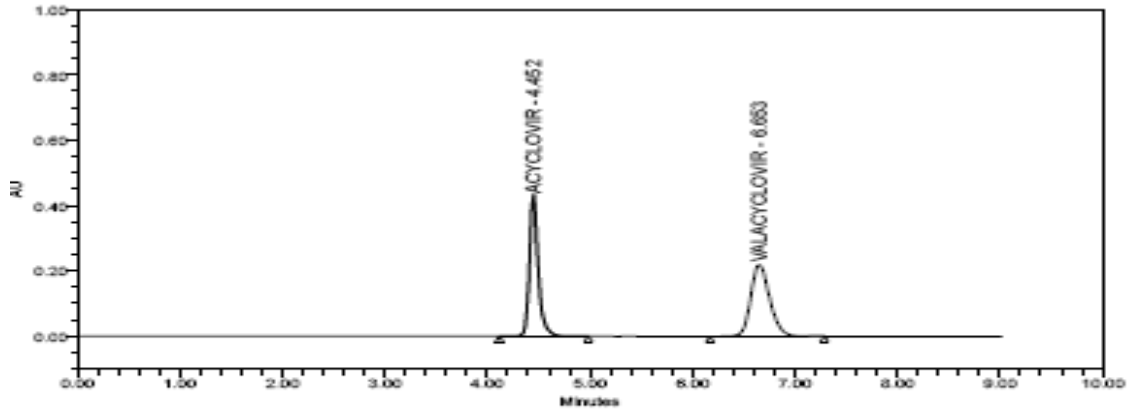
Stability in analytical solution: A solution of Acyclovir and valacyclovir were prepared and kept at room temperature. This solution was injected at intervals of 0, 2, 4, 8, 12, 16, 20 and 24hr. Area of all the Analytes were nearly identical to that obtained at 0h and additional peaks were not observed which indicate solution stability. The results are shown in Table 4.

Sample preparation of Acyclovir and Valacyclovir for routine analysis: Weighed 1 tablets of Zovirax (containing 200mg of Acyclovir) and 1 tablet of Valtrex (Containing 500 mg of Valacyclovir) sample in 100 ml volumetric flask, dissolved in diluents and dilute up the volume with diluents. Injected this solution into HPLC to determine the amount of analyte present in the sample. The chromatogram obtained after the analysis was shown in (Fig.5 to 7).



	Peak Name	RT	Area	% Area	Height
1	ACYCLOVIR	4.452	2593876	47.88	429086
2	VALACYCLOVIR	6.653	2824138	52.12	219698

Fig.6. Standard chromatogram of Acyclovir and Valacyclovir.



	Peak Name	RT	Area	% Area	Height
1	ACYCLOVIR	4.452	2593876	47.88	429086
2	VALACYCLOVIR	6.653	2824138	52.12	219698

Fig.7. Typical Sample chromatogram of Acyclovir and Valacyclovir.

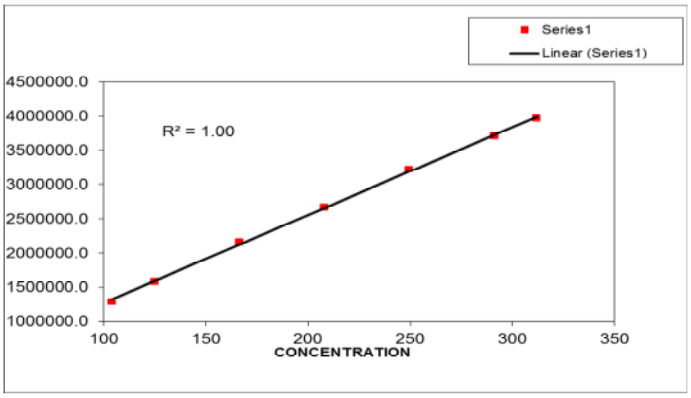


Fig.8. Linearity curve of Acyclovir.

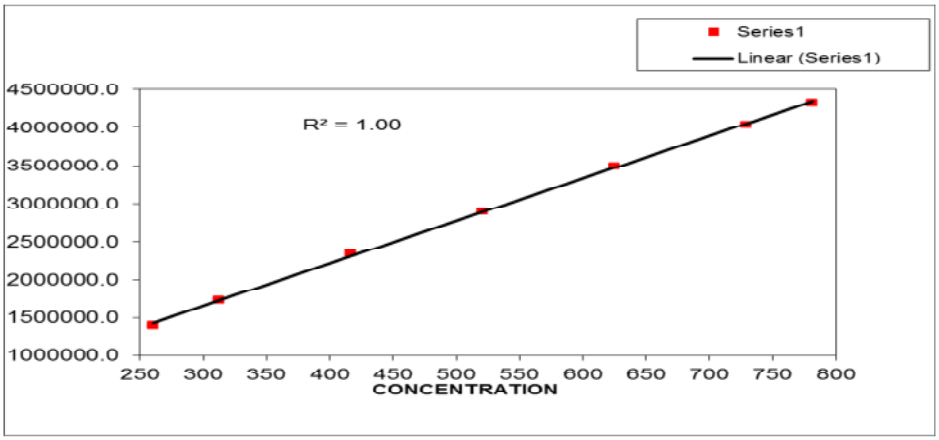


Fig.9. Linearity curve of Valacyclovir.

Force degradation: Sample prepared and treated as above given under the force degradation study condition as solid state (Elevated and photolysis) and liquid state (Acidic, Basic, Oxidation, Reduction and Hydrolysis) study and found that the assay of Acyclovir and Valacyclovir was within the limit. The results are shown in Table 5.

Table 5. Force Degradation Study: Recovery of Acyclovir and Valacyclovir.

Condition	Sample Area of Acyclovir	% Recovery of Acyclovir	Sample Area of Valacyclovir	% Recovery of Valacyclovir
Control	3189071	99.16	2403651	94.99
EV	3247062	100.97	2445863	96.66
Lux	3237368	100.66	2442162	96.51
Watt	3281661	102.04	2476492	97.87
Acidic 1Hr	2919073	90.77	2640459	104.35
Basic 1Hr 0.1M	2901547	90.22	2681637	105.98
Oxidation 2Hr	3247062	100.97	2445683	96.65
Reduction 1Hr	3112548	96.78	2752460	108.77
Hydrolysis 2Hr	3179852	98.88	2684972	106.11

Table 6. Force Degradation Study; Peak Purity of Acyclovir and Valacyclovir.

Condition	Acyclovir		Valacyclovir	
	PA	PT	PA	PT
Control	0.147	0.238	0.040	0.231
EV	0.212	0.253	0.054	0.256
Lux	0.166	0.241	0.055	0.235
Watt	0.200	0.248	0.052	0.251
Acidic 1Hr	0.215	0.254	0.045	0.242
Basic 1Hr 0.1M	0.125	0.218	0.048	0.247
Oxidation 2Hr	0.202	0.263	0.058	0.266
Reduction 1Hr	0.129	0.219	0.075	0.241
Hydrolysis 2Hr	0.149	0.241	0.041	0.245

The peak of Acyclovir and Valacyclovir was found pure for the above solid and liquid state degradation condition. The peak is pure when the peak angle is less than peak threshold. For results are shown in Table 6 and Fig. 10.

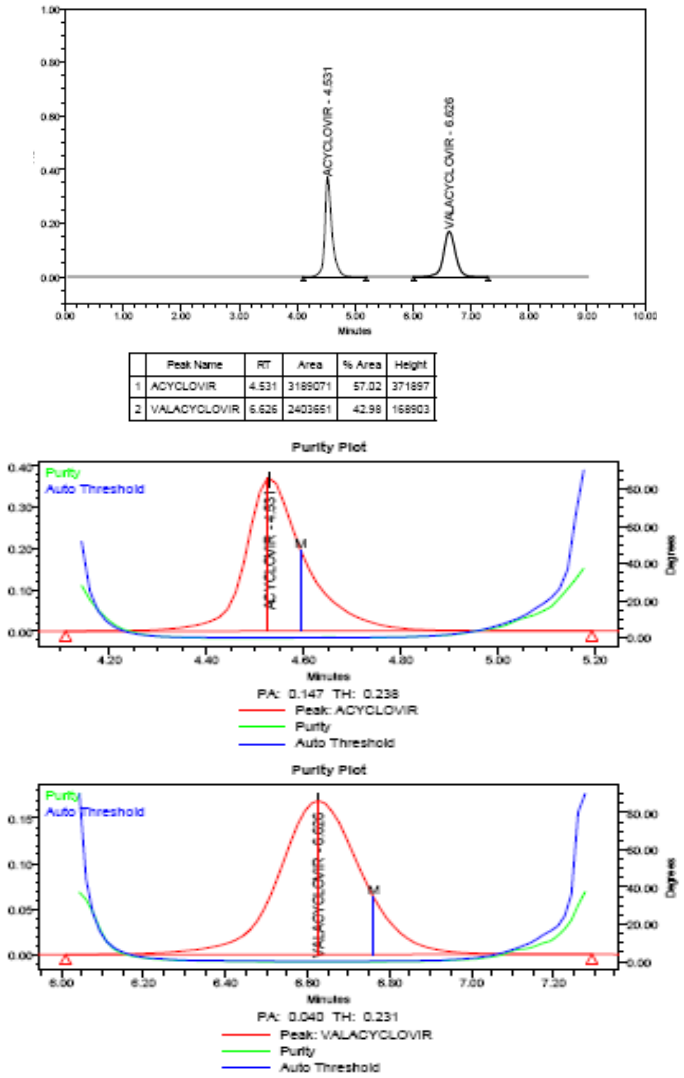


Fig.10. Control sample chromatogram of Acyclovir and Valacyclovir with peak purity table.

V. CONCLUSION

The proposed LC method is selective for the quantification of Acyclovir and valacyclovir present in zovirax and Valtrax. Hence this method is useful for the detection Acyclovir and valacyclovir in routine analysis.

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